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TITLE: Fyn: A Key Regulator of Metastasis in Prostate Cancer

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14. ABSTRACT My laboratory has continued to study the role of the novel molecular target, Fyn, in prostate cancer. In this second year of DoD funding we have made significant progress in defining the Fyn signaling pathway and its relationship with Met: a novel and important molecular target in several diseases including prostate cancer. Also, we have completed an important initial battery of murine studies showing an effect on dissemination: When Fyn deficient tumor cells were injected <i>in vivo</i> , their ability to metastasize was significantly diminished. Our studies continue to support the importance of Fyn as putative therapeutic target in advanced prostate cancer. This has become critically important given the recent report by Araujo of the long awaited phase 3 study of docetaxel with or without dasatinib, a Src-family kinase (SFK) inhibitor. The disappointing results have threatened the viability of applying SFK inhibition to the disease of prostate cancer. My group and others still believe that the completed study failed to translationally address the relevant biology of Fyn and SFKs in prostate cancer. Clinical studies with translational endpoints are now being planned with agents that effectively inhibit Fyn and Met in a variety of settings.					
15. SUBJECT TERMS- Fyn, Src, Met, prostate cancer, metastasis, dissemination, motility					
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INTRODUCTION:

Fyn is a 59-kDa member of the Src family of kinases (SFKs). Src and its family members are dysregulated in prostate cancer (CaP) and other malignancies. Our group identified Fyn as the most upregulated of this family in CaP and has set forward to describe its role in CaP. Our early data leading to this Idea award pointed toward the role of Fyn in directional cellular motility. The overall goal of this project is to test the underlying hypothesis that Fyn pathway upregulation augments directional cellular motility thereby, increasing CaP metastatic capacity and making it a relevant therapeutic target. These studies continue the work from our original CDMRP PCRP Physician Research Training Award by allowing them to evolve to the next level.

In our second year at Cedars-Sinai, our group continued to develop and characterize prostate cancer cell lines for understanding the role of Fyn in prostate cancer including a full set of PC3 and DU145 based constructs. During this year, Dr. Mink departed from our group and Dr. Murali Gururajan, an experienced CaP scientist, was recruited to my laboratory to continue and further these studies. Through these changes, we have met continued success in defining Fyn as a therapeutic target in prostate cancer.

SPECIFIC AIM 1. Quantify the impact of Fyn modulation on motility, directional velocity, and invasive capacity of prostate cancer cell lines in vitro.

A. Measure the impact of Fyn expression on directional motility and invasion in vitro.

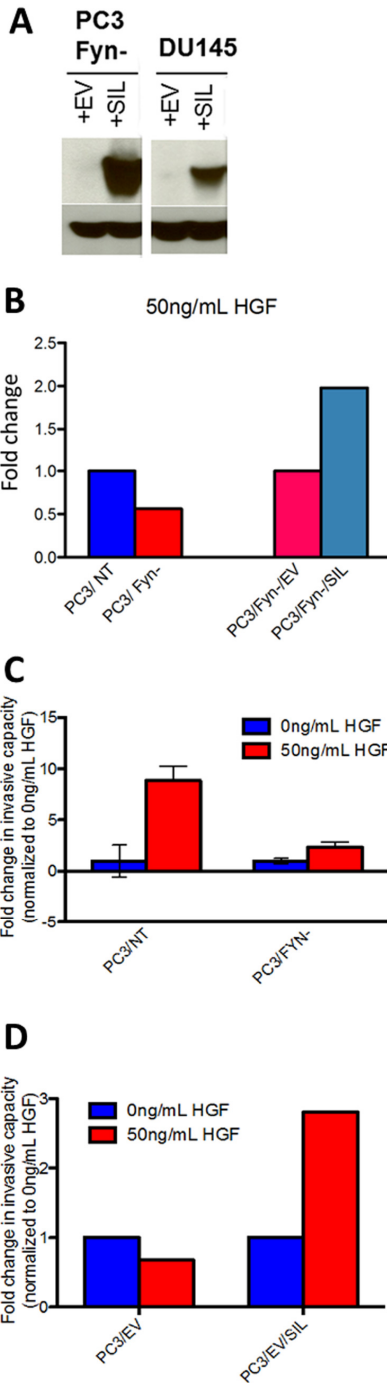


Figure 1. A) Fyn protein expression B) Growth and C&D) matrigel invasion assays of PC3/NT and PC3/FYN- and PC3/EV and PC3/EV/SIL cells.

Given issues with equipment during our reorganization, our laboratory resorted to traditional two-chamber and matrigel invasions assays to continue to study migration and invasion as related to Fyn expression. In particular, we successfully characterized the behavior of our PC3 and DU145 sublines: PC3/FYN-, PC3/NT, PC3/FYN-/SIL, and PC3/FYN-/EV as well as, DU145/EV and DU145/SIL (Figure 1A). The DU145 knockdown lines were not further characterized, as planned, given the relatively low levels of Fyn expression prior to knock down.

Figure 1 shows the growth characteristics of the PC3/FYN- and PC3/NT lines, as well as, the characteristics of the FYN-/EV (empty vector) and FYN-/SIL (rescued FYN-). We demonstrate that FYN- cells grows slower and FYN+ overexpressing cells grows faster than the control cells (Figure 1B). More importantly, it appears that Fyn promotes CaP invasion and migration *in vitro*, notably in response to HGF chemotactic gradients (Figure 1C&D). This relationship between Fyn and HGF has also been further explored as noted below.

B. Quantify the impact of alteration in Fyn expression on its signaling partners in vitro.

Given the slow progress with exploring candidate signaling partners based on literature reviews (including Pak1 which did not appear to be downstream of Fyn), a less biased approach to identify immediate binding partners of Fyn was pursued. A combination of a proteomic and transcriptomic approaches were used in form of immunoprecipitation with mass spectrometry and RNAseq (in collaboration with Dr. Jayoung Kim and Dr. Songyoung You- CSMC), respectively (Figure 2).

Using these techniques we identified potential binding partners including Met, plakoglobin, and cortactin. These proteins are involved in cytoskeletal organization and cellular motility- consistent with our observed phenotype.

As we had noted in our original tissue studies, suppression of Fyn expression also reduced activation of paxillin in response to HGF (verifying that paxillin is a substrate of Fyn).

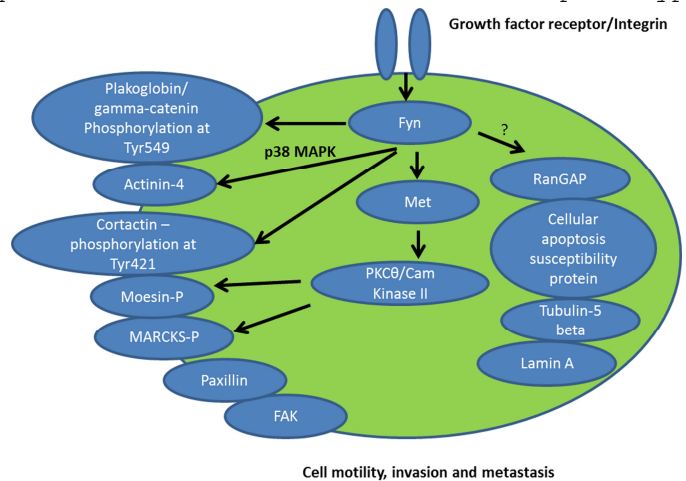


Figure 2. Modeling potential Fyn binding partners and substrates in prostate cancer cells based on results of mass spectrometry, immunoprecipitation, and RNAseq.

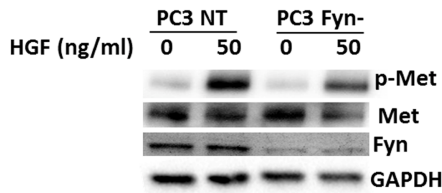


Figure 3. phospho-Met profile of PC3/NT and PC3/FYN- cells following HGF stimulation at 50 ng/mL for 20 minutes.

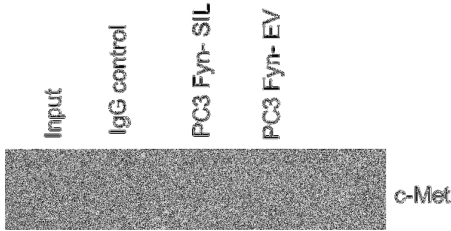


Figure 4. Met expression in PC3 lines PC3/FYN-/SIL vs. PC3/FYN-/EV

evident by detection of Met expression in lysates immunoprecipitated with Fyn. While our work demonstrated a relationship between Met and Fyn, we have been in need of a Fyn activation assay as well as an understanding of the biochemical events in human cancers. Our work suggests that Fyn facilitates Met phosphorylation and therefore hypothesized that reduced Fyn expression should increase the impact of a Met inhibitor. We tested this hypothesis in our PC3 sublines and found that Fyn- cells are more sensitive to Met inhibitors (Figure 5). We plan to broaden our work by testing a variety of Src kinase inhibitors that are in clinical trials in combination with Met inhibitors.

NEW DIRECTION: Fyn and Met. relationship between Fyn and Met using our Fyn-manipulated lines, we have found Fyn knockdown suppresses Met activation (independent of expression) (Figure 3). Upon overexpression of Fyn, Met phosphorylation returned. Expression of a kinase dead Fyn, however, failed to restore Met activity. As such,

we propose Fyn (like SRC) has the capacity to regulate Met activation. To further explore this relationship, we

performed an immunoprecipitation with anti-Fyn and probed for Met expression in PC3/EV/SIL (Figure 4). Interestingly, Fyn appears to physically interact with Met as

performed an enumeration of circulating tumor cells (CTCs) in the peripheral blood of Fyn- cells. We plan to broaden our work by testing a variety of Src kinase inhibitors that are in clinical trials in combination with Met inhibitors.

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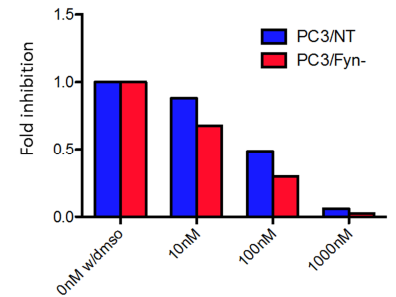
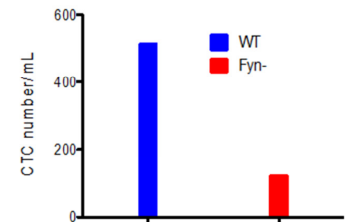


Figure 5. Growth assay of PC3/NT and PC3/FYN- cells in the presence of varied concentrations of c-Met inhibitor PF04217903



SPECIFIC AIM 2. To test the hypothesis that Fyn expression correlates with dissemination to and colonization of secondary (metastatic) sites in experimental metastasis models.

A. Measure the impact of Fyn expression on tumor cell dissemination from a given tumor volume in vivo.

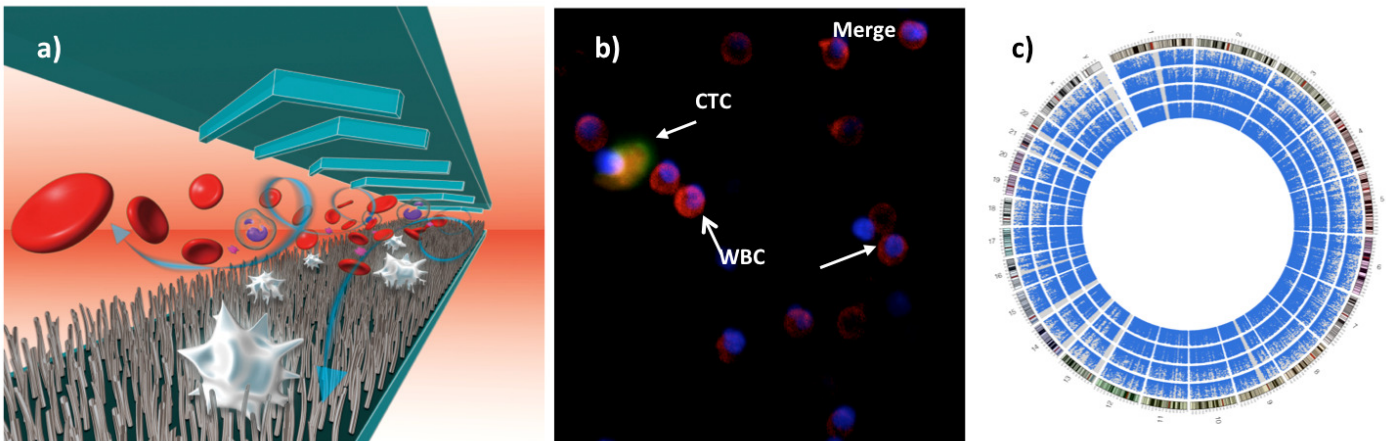


Figure 7. a) Conceptual illustration of NanoVelcro CTC Chip: a capture-agent (anti-EpCAM)-coated SiNW substrate is combined with an overlaid microfluidic chaotic mixer to enhance the contact frequency between CTCs and the SiNW substrate. b) Identification of CTCs based on a 4-color immunocytochemistry staining using FITC-conjugated anti-PSMA (green), PE-conjugated anti-CK (orange), Cy5-conjugated anti-CD45 (red, a marker for WBC) and DAPI (blue) was validated on a CSMC patient. c) Circos plots representing the coverage areas of whole exome sequencing. With each dot indicating a sequenced allele among the captured alleles by the exome-enrichment kit, the rings from the inside out represent WBC, pooled CTCs, CTC-1 and CTC-2. The outermost ring represents the karyotype of the human reference genome (hg19), with red areas being centromeres.

In the revised experiment, in addition to verifying our crystal violet findings, we made use of the human nature of the tumor cells and expression of human proteins on the cell surface. The blood from our mice was subjected to CTC capture using a NanoVelcro system developed jointly by my lab and Drs. Leland Chung (CSMC), Hsian-Rong Tseng (UCLA), and

Matthew Rettig (UCLA). Figure 6 shows representative data from these experiments using the mouse blood on our NanoVelcro system. Details of this system are given in figures 7 and 8. This system was brought into our laboratory through the aforementioned collaboration as a tool through which dissemination and metastasis could eventually be studied in human subjects. The work of this project has been crucial to developing this collaboration and will lead to additional metastasis investigations based on CTCs isolated from patients in the near future.

B. Quantify the change in end organ involvement related to decreased expression of Fyn after fixed tumor cell dissemination via intracardiac injection

My laboratory has developed luciferase expressing PC3 sublines (as described above). The luciferase-tagged cells were used in an intracardiac injection experiment coupled with xenogen imaging. To measure the impact of Fyn expression of invasive capacity of cells in mice, PC3 cells with and without the FYN knockdown were introduced into SCID mice via intracardiac injection. Mice were tracked by bioluminescent imaging using a luciferin substrate. At 6 weeks, we observed luminescent signals, lung metastasis, and death in the PC3/NT mice (Figure 9).

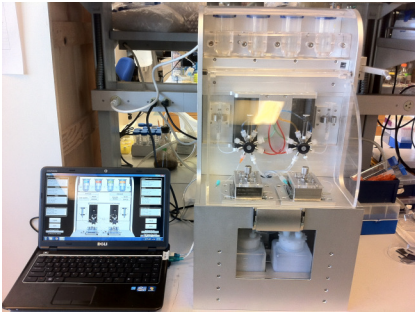


Figure 8. Automatic fluid handler that incorporates NanoVelcro CTC Chip

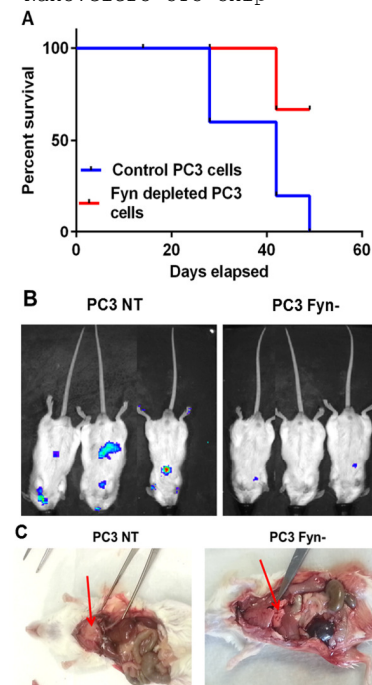


Figure 9. PC3 cells with and without the FYN knockdown tagged with a luciferase vector were introduced into SCID mice via intracardiac injection. A) Kaplan-Meier survival curve for mice injected with control versus Fyn knockdown cells B) Bioluminescent luciferase imaging using luciferin substrate at 6 weeks. C) Metastatic tumors visible in the lung in mice injected with the control cells but not the Fyn knockdown cells.

SPECIFIC AIM 3. Determine alterations of Fyn pathway members in human CaP and correlate them with demographic, pathological and clinical outcome parameters.

This aim remains under active development as Dr. Knudsen, director of the CSMC biorepository is completing the needed regulatory steps to allow us to gather tissue. Meanwhile we have opened an investigator-initiated and sponsored study of the potent Met inhibitor, cabozantinib in mCRPC with visceral disease from which we will obtain tissue and CTCs to further explore the relationship of Fyn expression and response to this agent. While this grant will not be used to conduct the trial, the preliminary data from our studies will be tested in biopsies obtained from our patients particularly with focus on the expression and activation of Fyn-kinase pathway members and their relationship to sensitivity (and/or toxicity) related to cabozantinib therapy.

During the conduct of this study, I closed a study of saracatinib (AZD0530) as a metastasis inhibitor in mCRPC. This heavily vetted study had a full accrual of 140 patients, but stopped early as one of the stopping rules was met. Given the use of this toxic agent in extremely late stage disease, patients often progressed clinically or were unable to tolerate the drug for a sufficient period of time to allow for the proper conduct of this clinical experiment. As such, it was deemed appropriate on my review of accrual to discontinue further accrual as the current design would not permit proper testing of the hypothesis that treatment with saracatinib would result in inhibition of Fyn and other SFKs resulting in decreased metastatic potential of mCRPC. Variations in response were seen and patients could be classified as potentially sensitive vs. insensitive to saracatinib. Tissue from many of these patients is available at the University of Chicago and will be shipped to my laboratory for analysis. Using our current findings, we will explore the expression and activation of Fyn-pathway members to determine if patterns of expression correlate with this dichotomy in the patient population.

Similarly, we have obtained tissue from a neoadjuvant study of dasatinib in locally advanced urothelial cancers. From these patients we have pre-treatment tissue from transurethral resection as well as blocks from the cystectomy. We are in

the midst of working with Dr. Knudsen to test these tissues for various markers including pSrc, Src, ki67, Akt, Erk, cleaved caspase 3, and others to determine if patterns of biomarker alterations were seen in relationship to dasatinib. This study resulted in no pathologic T0 findings, but we hope the biomarker inquiries will be useful.

Finally, in concert with Dr. Knudsen we are collecting blood for CTC analysis and profiling on our NanoVelcro platform. While we have pursued more global profiling using whole exome sequencing and/or whole genome sequencing on these CTCs, we continue to probe these data for findings related to Fyn, Met, and the Fyn pathway.

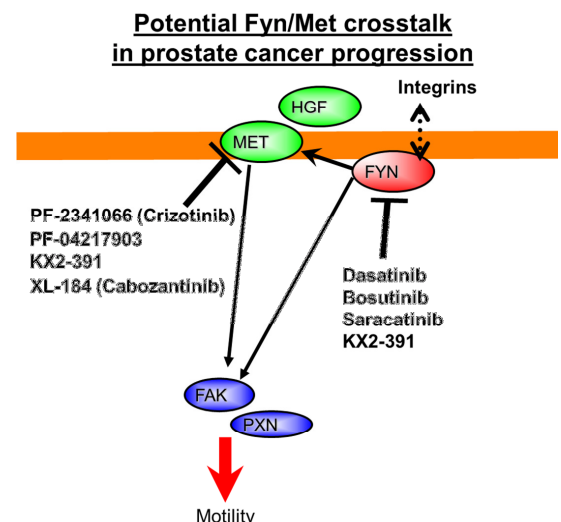
Key accomplishments

1. Recruitment of Dr. Murali Gururajan as a project scientist to support this research program. He replaced Dr. Sheldon Mink, research scientist I, in my group. Dr. Mink was not considered key personnel for this project. His departure did not adversely impact progress on or course of this project.
2. Progress with *in vivo* modeling work and developing additional
3. Recruitment of Dr. Murali Gururajan, Project Scientist and Dr. Yi-Tsung Lu, postdoctoral fellow to my laboratory group
4. Finding of Fyn as a regulator of Met activation
5. Finding of Met inhibition in Fyn knockdown cells as a sensitization approach.
6. Clinical study of saracatinib (AZD0530- a pan SFK inhibitor) as a metastasis inhibitor in advanced CRPC (PCCTC/CTEP sponsored) - completed and being reported (manuscript in preparation).
7. Translational phase II single arm study of cabozantinib (XL184) IST20 of XL-184 in advanced CRPC with visceral disease (open and accruing- tissues to be used for this research project.
8. Re-establishment of a translational prostate cancer research program at CSMC with Drs. Leland Chung, Michael Freeman, Neil Bhowmick, Beatrice Knudsen, Hyung Kim, Dolores DiVizio, Howard Sandler, and Stuart Holden

Future Directions

1. Dr. Gururajan and I are finalizing a manuscript to address the role of SFK inhibition in prostate cancer. Our data, and those from other groups, demonstrate the relevance of Src, Fyn, and other SFKs to this disease. The addition of SFK inhibition to docetaxel therapy may have been flawed in a true inability to build on docetaxel- a fact that has emerged in trial after trial in advanced prostate cancer. Even if this is the case, there remains an urgent need to identify key areas of development for SFK inhibitors and our forthcoming review will address those matters.
2. Development of Fyn-Met combination therapy in preclinical mouse models of human prostate cancer (Figure 9) and as a therapeutic strategy.
3. Characterization of Fyn and Fyn-pathway activation in circulating tumor cells (in collaboration with Drs. Leland Chung, Hsian-Rong Tseng, Haiyen Zhau, and Rouxiang Wang).
4. Characterization of Fyn and Src kinases as predictors of sensitivity to cabozantinib
5. Characterization of Fyn pathway in human tissue. At this time, we are planning a neoadjuvant study of bafetinib (FYN, LYN- inhibitor; Cytrix) at Cedars-Sinai.

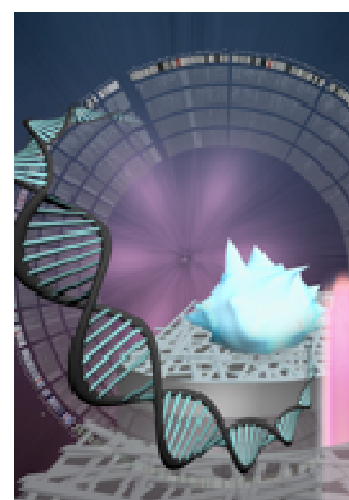
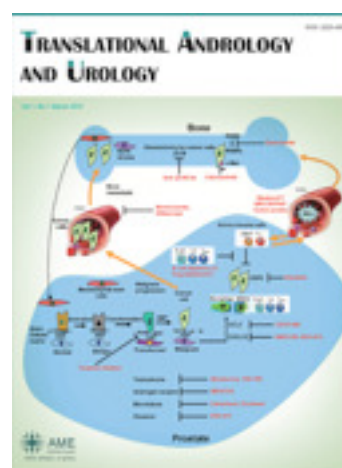
Figure 9. Combinatorial therapy targeting Fyn and Met in human prostate cancer.



Summary of additional progress

Recent publications

1. Jensen AR, David SY, Liao C, Dai J, Keller ET, Al-Ahmadie H, Dakin-Haché K, Usatyuk P, Sievert MF, Paner GP, Yala S, Cervantes GM, Natarajan V, Salgia R, **Posadas EM**. Fyn is downstream of the HGF/MET signaling axis and affects cellular shape and tropism in PC3 cells. Clin Cancer Res. 2011 May 15;17(10):3112-22. Epub 2011 Mar 1. (cover image)
2. Ward, J.E., Karrison, T., Chatta, G., Hussain, M., Shevrin, D., O'Donnell P, Szmulewitz, R.Z., Stadler, W.M., **Posadas, E.M.** "A randomized phase II study of pazopanib in castrate sensitive prostate cancer: a University of Chicago Phase II Consortium/ Department of Defense Prostate Cancer Clinical Trials Consortium study".
3. Gururajan M, **Posadas EM***, Chung LWK. Future perspectives of prostate cancer therapy. Trans Androl and Urol. *Co-corresponding author. Trans Androl and Urol 1:19-32 (2012)
4. Tannir NM, Wong Y, Kollmannsberger CK, Ernstoff MS, Perry DJ, Appleman LJ, **Posadas E.M.**, Cho D, Choueiri TK, Coates A, Gupta N, Pradhan R, Qian J, Chen J, Scappaticci F, Ricker JL, Carlson DM, Michaelson MD. Phase 2 Trial of Linifanib (ABT-869) in Patients with Advanced Renal Cell Cancer (RCC) after Sunitinib Failure. Eu J Cancer 47:2705-14
5. **Posadas EM**, Figlin RA "Systemic Therapy in Renal Cell Carcinoma: Advancing Paradigms". Oncology (Williston Park). Mar;26(3):290-301 2012.
6. **Posadas EM**, Figlin RA. Understanding the role of MET kinase in cancer therapy. J Clin Oncol. 2013 Jan 10;31(2):169-70.
7. Antonarakis ES, Heath EI, **Posadas EM**, Yu EY, Harrison MR, Bruce JY, Cho SY, Wilding GE, Fetterly GJ, Hangauer DG, Kwan MF, Dyster LM, Carducci MA. A phase 2 study of KX2-391, an oral inhibitor of Src kinase and tubulin polymerization, in men with bone-metastatic castration-resistant prostate cancer. Cancer Chemother Pharmacol. 2013 Jan 13.
8. Zhao L, Lu Y-T, Li F, Wu K, Hou S, Yu J, Shen Q, Wu, D, Song M, OuYang W-H, Luo Z, Lee T, Shao C, Xu X, Garcia MA, Chung LWK, Rettig M, Tseng H-R, and **Posadas EM***. High-Purity Prostate Circulating Tumor Cell Isolation by a Polymer Nanofiber-Embedded Microchip for Whole Exome Sequencing. * Corresponding author. **Advanced Materials**. Mar 26. doi: 10.1002/adma.201205237. [Epub ahead of print]
9. Lu Y-T, Zhao L, Shen Q, Garcia MA, Wu D, Hou S, Song M, Xu X, OuYang W-H, OuYang W-L, Lichterman J, Luo Z, Xuan X, Huang J, Chung LWK, Rettig M, Tseng H-R, Shao C and **Posadas EM*** .. NanoVelcro Chip for CTC enumeration in prostate cancer patients. * Corresponding author (**In press - Methods**)
10. Lu Y-T, Hou S, Zhao L, Lichterman J, Shen Q, Garcia MA, Wu D, OuYang W-L, Lichterman J, Luo Z, Xuan X, Huang J, Chung LWK, Rettig M, Tseng H-R, Shao C and **Posadas EM***. Next-generation Sequencing Reveals CTCs Sharing Recurrent Mutation Profiles with Primary and Metastatic Prostate Cancer Tissues . * Corresponding author (In preparation)
11. Gururajan M, Knudsen BS, Simons JW, and **Posadas EM***. Src kinase Biology and Therapeutics: Perspectives on clinical trials and outcomes. *Corresponding Author. (In preparation)



12. Gururajan M, Sievert M, and **Posadas EM***. Fyn kinase promotes prostate cancer growth and metastasis through activation of hepatocyte growth factor receptor.
*Corresponding Author. (In preparation)

Invited presentations

1. Prostate Cancer Therapy in 2012. Department of Medicine Grand Rounds, CSMC 2012
2. Multidisciplinary care in prostate cancer: the medical oncologists perspective. Cedars-Sinai Multi-disciplinary Cancer Management Program. Spring 2012.

Recruitment

Murali Gururajan, PhD

Yi-Tsung Lu, MD

Collaborations

- **Basic**
 - Cedars Sinai Medical Center:
 - Leland Chung, PhD- FYN/MET interaction
 - Chia-Yi Chu, PhD- *In vivo* metastasis assays of Fyn and Met
 - Neil Bhowmick, PhD- cysteine as a biomarker in PCa (forthcoming clinical trial)
 - Beatrice Knudsen, MD-PhD- MET/SRC/FYN interactions
 - Michael Freeman, PhD- FYN/DIAPH3 interactions and implications for shape and motility
 - Jayoung Kim, PhD- FYN related signaling
 - Songyoung You, PhD- FYN related signaling
 - Vancouver Prostate Centre
 - Amina Zoubedi, PhD- exploration of Fyn and AR regulation
 - Cleveland Clinic Foundation
 - Nima Sharifi, MD- exploration of AR biology in live CTCs
 - University of Michigan
 - Felix Feng, MD-PhD- Fyn and radiosensitivity;
- **Translational:**
 - Cedars-Sinai Medical Center
 - Leland Chung, PhD- CTC studies in PCa
 - Neil Bhowmick, PhD- cysteine as a biomarker in PCa (forthcoming clinical trial)
 - University of Michigan
 - Felix Feng, MD- RTOG phase 3 study with enzalutamide and RT
- **Clinical:**
 - Walter Stadler, MD- Randomized discontinuation study of AZD0530 (sarcatinib) in CRPC (Manuscript in preparation)
 - Leland Chung, PhD- (IRB approved) Phase II study of XL-184 in mCRPC
 - Felix Feng, MD- named as Med Onc PI for upcoming RTOG phase 3 study of enzalutamide in salvage radiotherapy for PCa

Promotions

UCLA/CSMC Associate Professor Appointment under review

Appointments

Medical Staff Leadership Program. Cedars-Sinai Medical Center (2011-2012)

Co-Medical Director: Urologic Oncology Center of Excellence- Cedars-Sinai Medical Center (2011-2012)

Cancer Quality Committee Member- Cedars Sinai Medical Center (2011-2012)

Protocol Review and Monitoring Committee Member (2011-2012)